Atrazine-Induced Hermaphroditism at 0.1 ppb in American Leopard Frogs (*Rana pipiens*): Laboratory and Field Evidence

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Atrazine is the most commonly used herbicide in the United States and probably the world. Atrazine contamination is widespread and can be present in excess of 1.0 ppb even in precipitation and in areas where it is not used. In the current study, we showed that atrazine exposure (≥ 0.1 ppb) resulted in retarded gonadal development (gonadal dysgenesis) and testicular oogenesis (hermaphroditism) in leopard frogs (*Rana pipiens*). Slower developing males even experienced oocyte growth (vitellogenesis). Furthermore, we observed gonadal dysgenesis and hermaphroditism in animals collected from atrazine-contaminated sites across the United States. These coordinated laboratory and field studies revealed the potential biological impact of atrazine contamination in the environment. Combined with reported similar effects in *Xenopus laevis,* the current data raise concern about the effects of atrazine on amphibians in general and the potential role of atrazine and other endocrine-disrupting pesticides in amphibian declines. *Key words:* amphibian, atrazine, endocrine disruption, hermaphrodite. *Environ Health Perspect* 111:568–575 (2003). doi:10.1289/ehp.5932 available via *http://dx.doi.org/* [Online 23 October 2002]

Atrazine is probably the most widely used herbicide in the world and one of the most common contaminants in ground and surface waters [U.S. Environmental Protection Agency (EPA) 1994]. Recently, Tevera-Mendoza et al. (2002) showed that atrazine exposure (21 ppb) for as little as 48 hr resulted in severe gonadal dysgenesis in African clawed frogs (*Xenopus laevis*). Further, we showed that atrazine induced hermaphroditism at concentrations of only 0.1 ppb (Hayes et al. 2002) when administered throughout larval development. Most water sources in the United States, including rainwater, can exceed the effective concentrations in these laboratory studies (Hayes et al. 2002). In addition, the concentration in our previous study (Hayes et al. 2002) is 30 times lower than the current drinking water standard (Hayes 1993). Despite the significance of the reported effects in *X. laevis*, both studies (Hayes et al. 2002; Tevera-Mendoza et al. 2002) were conducted in the laboratory on a single species; whether the effects of atrazine are widespread in amphibians and whether effects occur in the wild remained unanswered.

In the current study, we examined the effects of atrazine on leopard frogs (*Rana pipiens*), a U.S. native species, under controlled laboratory conditions. Once effects of atrazine were identified, we examined wild *R. pipiens* from a variety of habitats in areas with reportedly low atrazine use and areas with high atrazine use in a transect that extended from Utah to the Iowa–Illinois border. Further, we collected water samples and examined atrazine contaminant levels at each site. These coordinated laboratory and field analyses uniquely addressed the ecological significance and relevance of our initial laboratory studies.

Materials and Methods

Animal care for laboratory studies. Leopard frogs (*R. pipiens*) were obtained from Sensiba Marsh, Brown County, Wisconsin, and shipped overnight to the University of California at Berkeley. Eggs were allowed to hatch and then were apportioned into rearing tanks. Larvae (30/tank) were reared in 4 L aerated 10% Holtfreter's solution (Holtfreter 1931) and fed Purina rabbit chow (Purina Mills, St. Louis, MO). Food levels were adjusted as larvae grew to maximize growth. Experiments were carried out at 22–23°C with animals under a 12-hr light/12-hr dark cycle (lights on at 0600 hr).

Larval laboratory exposures. Larvae were treated by immersion with nominal concentrations of 0, 0.1, or 25 ppb atrazine (98% pure; Chemservice, Chester, PA). Concentrations were confirmed by chemical analysis. Atrazine was predissolved in ethanol, and all treatments contained 0.0036% ethanol. Each treatment was replicated three times (30 larvae/replicate). Cages were cleaned, water changed, and treatments renewed every 3 days. All treatments were systematically relocated every 3 days to ensure that no treatments or tanks experienced position effects. Animals were exposed throughout the larval period from 2 days posthatching until complete tail reabsorption. In all experiments, all dosing and analyses were conducted blindly with color-coded tanks and treatments.

General measurements. At metamorphosis (complete tail reabsorption), each animal was weighed and measured. Animals were euthanized in 0.2% benzocaine (Sigma Chemicals, St. Louis MO), assigned a unique identification number, fixed in Bouin's fixative, and preserved in 70% ethanol until further analysis.

Histological analysis of gonads. All analyses were conducted blindly. Initially, the sex of all individuals was determined based on gross gonadal morphology using a Nikon SMZ 10A dissecting scope (Technical Instruments, Burlingame, CA). In the laboratory study, histological analysis was conducted on nine females per treatment and on all males. All histology was conducted according to Hayes (1995). In brief, tissues were dissected and dehydrated in graded alcohols followed by infiltration with Histoclear and paraffin (National Diagnostics, Atlanta, GA). Serial histological sections were cut at 8 µm through the entire gonad. Slides were stained in Mallory's trichrome stain and analyzed using a Nikon Optiphot 2 microscope (Technical Instruments). Images of gonads were recorded using a Sony DKC-5000 digital camera (Technical Instruments). For gonadal analysis, we examined every section from each gonad.

Site selection for field studies. Initially, we chose study localities based on atrazine use, as determined by atrazine sales (Figure 1). All localities were between 39°N and 43°N latitude. Counties with < 0.4 kg/km^2 atrazine use were chosen as potential control sites, and areas with > 9.3 kg/km^2 atrazine use were chosen as potential atrazine-exposed sites. We began sampling in Utah on 15 July 2001 and moved eastward. In Utah, we chose one site (Juab County) in an area with < 0.4 kg/km^2 atrazine sales, and we collected in Cache County, Utah, with 0.4–2.4 kg/km^2 reported atrazine use. Carbon County, Wyoming, was considered a control site because the locality is not in the vicinity of farms, and the county (most of the

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